PERSPECTIVE AND HYPOTHESIS

Animal Egg as Evolutionary Innovation: A Solution to the "Embryonic Hourglass" Puzzle

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STUART A. NEWMAN*
Department of Cell Biology and Anatomy, New York Medical College, Valhalla, New York

ABSTRACT

The evolutionary origin of the egg stage of animal development presents several difficulties for conventional developmental and evolutionary narratives. If the egg's internal organization represents a template for key features of the developed organism, why can taxa within a given phylum exhibit very different egg types, pass through a common intermediate morphology (the so-called "phylotypic stage"), only to diverge again, thus exemplifying the embryonic "hourglass"? Moreover, if different egg types typically represent adaptations to different environmental conditions, why do birds and mammals, for example, have such vastly different eggs with respect to size, shape, and postfertilization dynamics, whereas all these features are more similar for ascidians and mammals? Here, I consider the possibility that different body plans had their origin in self-organizing physical processes in ancient clusters of cells, and suggest that eggs represented a set of independent evolutionary innovations subsequently inserted into the developmental trajectories of such aggregates. I first describe how "dynamical patterning modules" (DPMs) associations between components of the metazoan developmental-genetic toolkit and certain physical processes and effects may have organized primitive animal body plans independently of an egg stage. Next, I describe how adaptive specialization of cells released from such aggregates could have become "proto-eggs," which regenerated the parental cell clusters by cleavage, conserving the characteristic DPMs available to a lineage. Then, I show how known processes of cytoplasmic reorganization following fertilization are often based on spontaneous, self-organizing physical effects ("egg-patterning processes": EPPs). I suggest that rather than acting as developmental blueprints or prepatterns, the EPPs refine the phylotypic body plans determined by the DPMs by setting the boundary and initial conditions under which these multicellular patterning mechanisms operate. Finally, I describe how this new perspective provides a resolution to the embryonic hourglass puzzle. *J. Exp. Zool. (Mol. Dev. Evol.) 316:467–483, 2011.* © 2011 Wiley Periodicals, Inc.

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> Nor does he begin the Trojan War from the egg, but always he hurries to the action, and snatches the listener into the middle of things.
>
> - Horace

The eggs of animal species present a remarkable variety of sizes, shapes, and interior patterns and patterning processes. Animal eggs may be yolky, nonyolky, isolecithal (yolk evenly distributed), telolecithal (yolk unevenly distributed), or centrolecithal (yolk centrally placed), with or without maternal determinants, capable or not of sustaining postfertilization spatiotemporal calcium transients, employing microfilaments or microtubules in rearranging precleavage cytoplasm. Strikingly, these eggs types are not found uniquely in specific phyla. The same phylum can present many varieties of eggs, and contrarily, phylogenetically distant taxa can exhibit morphologically similar egg types (Gilbert, 2010).

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Abbreviations: DPM, dynamical patterning module; EPP, egg-patterning process.

*Correspondence to: Stuart A. Newman, Department of Cell Biology and Anatomy, New York Medical College, Valhalla, New York.
E-mail: newman@nymc.edu

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Organisms of the different vertebrate classes, for example, start off as eggs that may differ in shape and size by several orders of magnitude. In a similar fashion, closely related nematode species can have drastically different cleavage patterns, despite converging on virtually indistinguishable late embryonic morphologies. Certain of these nematode cleavage pathways, moreover, resemble those of some vertebrates more than they do those of their sister nematode species (Schierenberg, 2006).

Plausible evolutionary scenarios do not require embryos to closely track ancestral forms or to generally evolve by addition to the terminal stages of embryonic development. Within individual phyla or subphyletic taxa, however, embryos often converge on a common morphological platform from which subsequent diversification took place. In the case of the vertebrates, for example, that platform, usually considered to be the "pharyngula" stage (Ballard, '81; see also Galis and Metz, 2001), existed for tens of millions of years between its appearance with the first craniates and the emergence of the first tetrapods. But, the pharyngula stage in modern amphibian, avian, and mammalian species is arrived at by vastly different routes from strikingly different eggs.

In insects, although it is easy to distinguish between adult stages of the grasshopper *Schistocerca* and the fruit fly *Drosophila*, their germ band stages look remarkably similar morphologically and even in terms of spatiotemporal expression of many orthologous genes. Nonetheless, the developmental events preceding the germ band stage differ markedly between the two insect genera. Grasshoppers employ the short germ band mode of segmentation, with their body segments budding off in a sequential manner from a posterior growth zone of a cellular blastula. In contrast, fruit flies are long germ band insects, forming their segments simultaneously from an originally syncytial blastula, and much faster than in grasshoppers (reviewed in Damen, 2007).

The passage through a morphologically conserved intermediate stage of development in vertebrates before they go on to assume their class-specific characteristics has been termed the "embryonic hourglass" (Duboule, '94; Raff, '96; Hall, '97; earlier recognition of this phenomenon by Ernst Haeckel, Joseph Needham and others is discussed in Horder, 2008). The hourglass format (Fig. 1A) or variations thereof, in which morphological disparity in a phylum's developmental trajectories is more pronounced before or after a conserved stage (Salazar-Ciudad, 2010), pertains to many, if not all, animal phyla. (For brevity, all such cases will be referred to as the "hourglass.") The narrow neck of the hourglass for each phylum has often been termed the "phylotypic stage" (Sander, '83), although this designation has been questioned (Wray and Strathmann, 2002; Bininda-Emonds et al., 2003; Poe and Wake, 2004).

This article is concerned with the preconstriction portion of the hourglass (the lower portion of Fig. 1). (For a discussion of the determinants of the postconstriction portion, see Salazar-Ciudad, 2010.) Cases, such as nematodes, vertebrates, annelids (Shimizu, '99), in which wide disparity at early embryonic stages is succeeded by a convergent morphotype at later stages raise the question of why some phylum-characteristic anatomical motifs, though transient, are nonetheless conserved. Proposed answers include constraints arising from the functional requirements of embryonic life (Wray and Strathmann, 2002), stabilizing selection protective of highly integrated mid-stage developmental processes against disruptive effects (Raff, '96; Galis and Sinervo, 2002; Galis et al., 2002), inherent robustness of certain mid-developmental mechanisms (von Dassow et al., 2000), and the observation that evolutionary change is easier between ontogenetically adjacent events (Poe and Wake, 2004). None of these earlier analyses has considered the possibility as does the one presented here that events in the egg may have only a modest impact on the body plan, because the key processes of morphological development are not operative until the embryo achieves a critical spatial scale and cell number (although Salazar-Ciudad, 2010, makes an analogous point regarding events at the blastula stage).

The evolution of multicellularity, the origin of eggs, and the basis of conserved embryonic stages have usually been discussed in gene-centered terms (Horder, 2006). Such analyses have been concerned with issues such as pleiotropy, modularity, and adaptive penetrance (e.g., Galis and Metz, 2001; Galis and Sinervo, 2002; Galis et al., 2002). Other work has focused on the dynamics of conflict, competition, and cooperation, e.g., between motility and differentiation, different lineages, and between forms derived from different kinds of eggs (Buss, '87; Grosberg and Strathmann, '98; Kerszberg and Wolpert, '98; Michod and Roze, 2001). Here, I make a proposal that places physical determinants of biological form at the center of a combined theory of the origin of eggs and the embryonic hourglass. Focusing on the fact that living tissues, by virtue of their intrinsic physical properties, are capable of self-organization, I argue that the emergence of phyletic diversity in the animals did not depend on the prior evolution of an egg stage of development. More specifically, primitive versions of phylotypic body plans could have arisen in cell clusters that arose from cell aggregation rather than the cleavage of eggs (Fig. 1B).

How were developmental programs propagated from one generation to the next in the absence of a gamete stage? Here, the analogy to *Dictyostelium discoidum*, a social amoeba with a free-living stage and several multicellular ecophenotypes, is helpful (Bonner, 2009). This organism has no gametes, but its cells are capable of aggregating under appropriate conditions and responding to environmental and intra-aggregate cues so as to differentiate, exhibit a "division of labor," and undergo morphogenesis. Any amoeba under the appropriate conditions can reconstitute the entire developmental sequence or "life cycle," as can aggregates of genetically heterogeneous amoebae (Nanjundiah and Sathe, 2011).

In the view discussed here, the ancestral prototypes of the metazoan phyla were direct descendents of free-living cells (likely related to the present-day choanozoans) which, under new

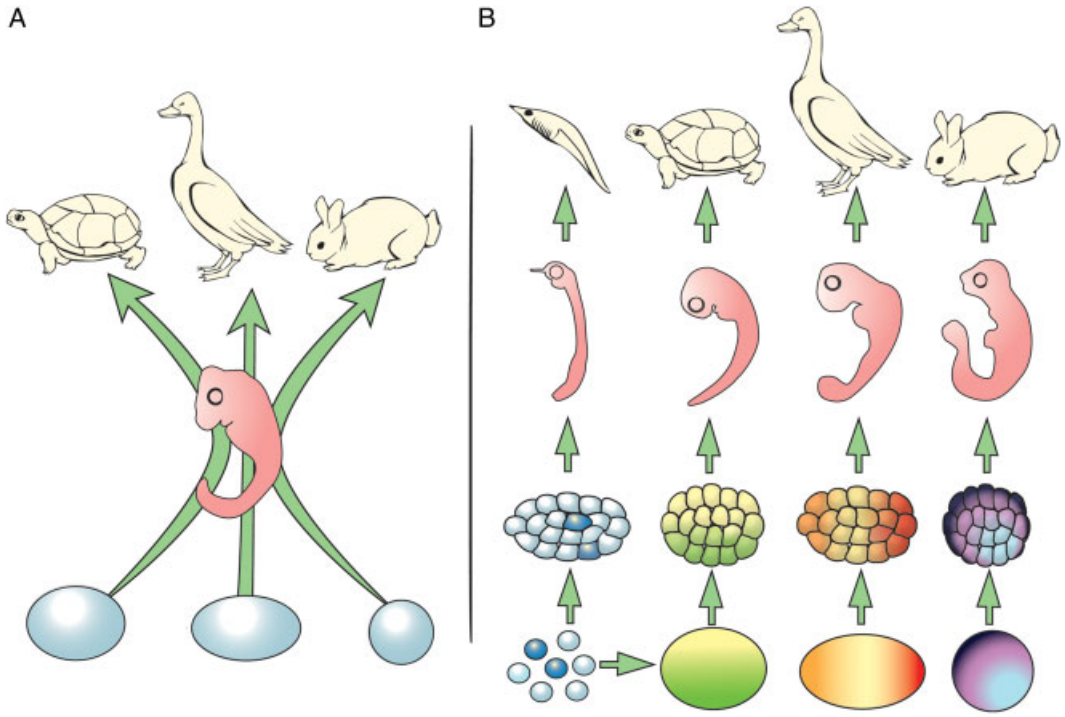

Figure 1. Classical and revised views of the "embryonic hourglass." (A) Standard representation of the embryonic hourglass. Morphologically different eggs of classes or other categories of organism in a given phylum converge developmentally on a conserved phylotypic morphology before diverging in form during later stages of development. One or another of the egg forms is considered to be primitive, with other forms evolving from it coordinately with the diversification of the subphylum taxa owing to natural selection. The example shown is of vertebrates, the conserved phylotypic stage is the pharyngula. (B) Proposed revised interpretation of the hourglass phenomenon. Each phylum originates from the action of a specific set of dynamical patterning modules (DPMs) on aggregates of similarly sized choanozoan-like founder cells—the "morphogenetic stage" of the phylum (a hypothesized primitive chordate is shown as an example). Other than expressing the phylum characteristic set of DPM-associated toolkit genes, not all the cells are genetically identical. Enlargement or reshaping of a founder cell (proposed to have happened independently in different phyla, but as transformations of a proto-egg in subphylum lineages, as in the example shown) activates one or more egg-patterning processes (EPPs), which leads to molecular nonuniformities within the cytoplasm of the founder cell. Cell division or cleavage of this proto-egg (and later egg) regenerates the morphogenetic stage, but now with a patterned (e.g., axial polarized) distribution of cells, which are now, however, genetically identical. The operative DPMs are the same phylum—characteristic ones as were present before the innovation of the egg stage. This is reflected in the resulting organisms being variations on a common body plan. The reproducible polarities and heterogeneities of the morphogenetic stage when it derives from an egg stage enable the DPMs to generate more reliable and stereotypical developmental outcomes, because the boundary and initial conditions of their action are less subject to stochastic effects.

circumstances, aggregated into clusters owing to the capacity of preexisting cell surface cadherins (Abedin and King, 2008) to mobilize the physical force of adhesion. Once this had occurred, the products of other genes present in the ancestral cells, acting in this new context (i.e., the increased spatial scale of the multicellular aggregates and independent mobility and proximity of the constituent cells), automatically mobilized additional physical processes and effects capable of mediating morphogenesis and pattern formation (Forgacs and Newman, 2005), and in this way generated each incipient phylotype (Newman et al., 2006; Newman and Bhat, 2008, 2009).

By this hypothesis, the "rough drafts" of the metazoan phyla in the Precambrian and early Cambrian would have been persisting colonies of single-celled organisms that had acquired the capability to undergo morphogenesis and pattern formation (Maynard Smith and Szathmáry, '95). Because of the role of physics in organizing these entities, there is no need to assume that they acquired their forms by incremental rounds of natural selection. Such organisms would have had simple gameteless life-cycles with their developmental processes becoming reconstituted each time cells that had detached from the colonies aggregated to form a new colony.

I suggest that specific types of eggs arose later, as enlarged, reshaped, and armored modifications of the colonial cells. The first step in the evolution of eggs would have been the emergence of "proto-eggs," as adaptations or accommodations of liberated cells to varied environments. However they originated, proto-eggs would have been subject to spontaneous cytoplasmic reorganization owing to another set of physical processes distinct from those operating in the multicellular context, which were set into motion when single-cell functionalities came to operate in cells of changed size and shape (Newman, 2009).

Regardless of the sizes, shapes, coatings, content of stored nutrients, and cytoplasmic inhomogeneities of proto-eggs and later true eggs, their capacity to regenerate the organisms that spawned them required that they return to the colony stage. This typically (but not invariably) occurs by cleavage. The subdivision of these large cells thus represented an alternative (i.e., nonaggregative) route to multicellularity.

In the remainder of this article, using the concept of "dynamical patterning modules" (DPMs) (Newman and Bhat, 2008, 2009), I describe in more detail the scenario in which animal body plans can have originated in the absence of an egg stage of development. Following this, I show how proto-eggs were plausibly inserted as developmental novelties into the life-cycles of such organisms. Next, I show how these putative proto-eggs might have assumed the character of present-day eggs, when preexisting cell physiological processes were mobilized in cells of changed size and shape. This change of context, I will argue, unleashed various self-organizational "egg-patterning processes" (EPPs), without undermining the phylotypic morphogenesis of the respective organisms. Finally, I show how these ideas, taken together, can resolve the hourglass puzzle.

DYNAMICAL PATTERNING MODULES

Morphogenesis and pattern formation in metazoan embryos are mediated to a great extent by the ability of certain gene products or their derivatives to mobilize specific physical processes that act on viscoelastic materials of the "middle" (100 μm–10 mm) scale (Forgacs and Newman, 2005). The presence of one or another of these molecules will vary with the phylum, but if a given one is present, so will the associated physical effects. The resulting organizing principles can be schematized into a dozen or so DPMs (Newman and Bhat, 2008, 2009). The DPM-facilitating molecules are a cell–cell interaction and signaling subset of the "developmental genetic toolkit": cadherins, components of the Wnt and Notch pathways, Hedgehog, BMPs and other morphogens, extracellular molecules, such as chitin and collagen, and various receptors. Most, if not all, of these "interaction toolkit" molecules were present in common ancestors of the Metazoa and the Choanozoa, the latter a unicellular sister phylum of the animals (King et al., 2008; Shalchian-Tabrizi et al., 2008; Sebé-Pedrós et al., 2010) (Table 1).

The phenomenon of multicellularity opened up possibilities for these molecules to become involved in the molding of bodies and organs. This was because certain individual cell properties mediated by these molecules, including adhesion, shape and surface polarization, switching between alternative biochemical states,

Table 1. Major metazoan dynamical patterning modules (DPMs).

DPM	Molecules	Physics	Evo–devo role
ADH[a]	Cadherins; lectins	Adhesion	Multicellularity; tissue formation
LAT	Notch pathway	Lateral inhibition	Coexistence of alternative cell types
DAD	Cadherins; lectins	Differential adhesion; phase separation	Tissue multilayering
POL_a	Catenin-associated Wnt pathway	Cell surface anisotropy	Topological change; internal cavities
POL_p	Catenin-independent Wnt pathway	Cell shape anisotropy	Tissue elongation
ECM	Chitin; collagen	Stiffness; dispersal	Skeleton formation; elasticity; EMT
OSC	Hes+Notch; Wnt	Synchronization of cell state	Developmental fields; periodic spatiotemporal patterning
MOR	Hh; TGF-β/BMP	Diffusive transport	Spatial patterning
ASM	FGFs and FGFRs	Reciprocal binary interaction	Induction
TUR	Hh; TGF-β/BMP+Notch	Dissipative structure	Periodic spatial patterning
MIT	MAPK	Mass increase	Tissue growth
APO	TNF; TNFR; Bcl-2; SMACs	Mass decrease	Tissue loss

[a]Acronyms for the DPMs: ADH, cell–cell adhesion; LAT, lateral inhibition; DAD, differential adhesion; POL_a, (multicellular) apicobasal polarity; POL_p, (multicellular) planar cell polarity; ECM, (multicellular) extracellular matrix; OSC, (multicellular) oscillation; MOR, morphogen; ASM, asymmetric interaction; TUR, Turing-type reaction-diffusion process; MIT, (multicellular) mitogenesis; APO, (multicellular) apoptosis. Acronyms for molecules: Hh, hedgehog; TGF-β, transforming growth factor-β; BMP, bone morphogenetic protein; FGF, Fibroblast growth factor; FGFR: fibroblast growth factor receptor; MAPK, mitogen-activated protein kinase; TNF, tumor necrosis factor; TNFR, tumor necrosis factor recptor; Bcl-2, B-cell lymphoma 2 apoptosis regulator; SMAC, second mitochondria-derived activator of caspases. Based on Newman and Bhat (2008, 2009).

biochemical oscillation, and secretion of diffusible and non-diffusible factors, have novel global consequences when they are manifested in multicellular aggregates (Newman and Bhat, 2008).

When multicellularity first arose at the origin of the metazoans by deployment of the first DPM–intercellular adhesion–a whole train of other middle-scale phenomena was set in play: (i) tissue multilayering owing to the phase separation associated with differential adhesion; (ii) lumen formation resulting from cell-surface polarization; (iii) tissue elongation (often via "convergent extension") resulting from cell shape polarization; (iv) local cell type heterogeneity resulting from lateral inhibition of cell state switching; (v) spatial variation in cell state across a tissue mass resulting from effects of morphogen gradients or reaction–diffusion processes; (vi) morphogenetic field formation (i.e., global coordination of cell state across a tissue mass), as a consequence of synchronization of intracellular oscillations; (vii) segmentation of tissue masses by interaction of gradients, synchronized cell state oscillations, and growth; and (viii) invaginations and evaginations of cell sheets owing to elastic properties of secreted extracellular matrices.

The composition, physics, and developmental roles of DPMs are described in detail in earlier publications (Newman and Bhat, 2008, 2009; Newman et al., 2009). Here, I note two points which are relevant for the proposed solution to the hourglass puzzle: (i) DPMs can organize cell aggregates without the need for any prespecification in the egg, and (ii) a given set of DPMs can produce slight variations on the same basic plan owing to their sensitivity to the initial and boundary conditions. The first point speaks to the capabilities ancient cell clusters possessed despite the absence of an egg stage of development (thus, the possibility that eggs represented an evolutionary innovation). The second point addresses the basis of the robustness of the morphological phylotype despite the diversity of eggs and their variation once egg stages were incorporated into development.

Depending on the genomes of their single-celled progenitors, metazoan cell clusters would have expressed overlapping but partly different DPM-associated molecules, and therefore have embodied different sets of DPMs. The different phyla, in fact, can be generally characterized by their DPMs (Table 2). Although the earliest radiating diploblastic metazoan phyla lack one or another of the basic DPMs (Srivastava et al., 2008, 2010), the various (later-appearing) triploblastic phyla seem to contain all of the DPMs, but differ from one another in the molecular and physical natures of their extracellular matrices.

Different phyla may also have acquired alternative developmental pathways over the course of evolution, owing to the nonlinear nature of the DPMs they have in common. Because markedly different outcomes of a dynamical system can be triggered by small differences in initial conditions (the "butterfly effect"), one may speculate that dorsoventral axis inversion between arthropods and chordates (Geoffroy Saint-Hilaire, 1822; Arendt and Nübler-Jung, '94, '99a,b; De Robertis and Sasai, '96) or repositioning of the mouth relative to the BMP-chordin axis between the hemichordates and chordates (Lowe et al., 2006) may have been instigated by switching between alternative dynamical modes in originally similar systems.

In addition, as mentioned above, the developmental outcomes of each of the DPMs are both robust to variations in and (subject to these limits) sensitive to the boundary and initial conditions of the systems in which they operate. Two populations of cells, for example, can remain intermixed if the number of cell adhesion molecules on their surfaces are relatively similar, but will sort out (a kind of phase separation) if these numbers exceed a threshold (Steinberg and Takeichi, '94). Reaction–diffusion processes, to take another example, generate patterns (e.g., regularly spaced spots) that can remain unchanged under small variations in domain shape, temperature, or other system parameters, and then abruptly switch over to other pattern modes (e.g., stripes) when certain threshold values are passed (Kondo and Miura, 2010; Zhu et al., 2010).

The sensitivity of such patterning processes to initial conditions can be utilized during evolution to yield more reliable outcomes. A cluster of cells, each one having the same number of

Table 2. Presence of selected DPMs and distinctive body plan features in different animal phyla.

Phylum	DPMs	Body plan features
Placozoa	ADH; DAD; MOR; POL_a; ECM[a]	Nonintermixed layers of uniform cell types
Porifera	ADH; DAD; MOR; POL_a; LAT; ECM[b]	Mixed cell types arranged around labyrinthine lumens
Cnidaria	ADH; DAD; MOR; $POL_{a,p}$; LAT; ECM[c]; ASM	Nonintermixed layers of mixed cell types
Ecdysozoa (Arthropoda; Nematoda)	ADH; DAD; MOR; $POL_{a,p}$; LAT; ECM[d]; ASM	Nonintermixed layers of mixed cell types; exoskeleton
Chordata	ADH; DAD; MOR; $POL_{a,p}$; LAT; ECM[e]; ASM	Nonintermixed layers of mixed cell types; endoskeleton

[a]Basement membrane ECM.
[b]Interstitial ECM.
[c]Basement membrane ECM.
[d]Basement membrane and cuticular ECM.
[e]Basement membrane and interstitial ECM.

adhesive proteins on its surface, will form a homogeneous sphere. If, however, subsets of cells in the cluster express either high or low amounts of this same adhesive molecule (as in the example in the previous paragraph), spontaneous sorting owing to differential adhesion (the DPM referred to as "DAD"; Newman and Bhat, 2008, 2009) will lead to a multilayered structure with a predictable inside–outside arrangement (Steinberg, 2007). It does not matter whether the adhesive differences arise randomly or are prespecified, possibly owing to an asymmetrical arrangement of some molecular cue in the cluster's founding cell; in either case, a similar bilayered arrangement will form. This illustrates the robustness of determination by DPMs.

In the "random" case, however, in which the cells that end up in the different layers are not predetermined to do so, any cell's subsequent differentiated fate, say as a neural derivative of the outer layer, would have to arise de novo in that cell. In the "prespecified" case, in contrast, differentiation-determining factors, e.g., for a neural fate, can be conveyed into cells of the appropriate layer simply by colocalization in the egg with the determinants of the adhesive differentials. This illustrates that initial conditions can make a difference in allocating specific cells to one or another layer, and that this can have functional utility.

A second example relates to cases in which the Notch pathway is employed to enforce a lateral inhibitory effect (the DPM termed "LAT"; Newman and Bhat, 2008, 2009). This enables a cell within a cluster, which begins to differentiate along a certain pathway, to cause cells adjacent to it to develop along an alternative route. If all the cells are initially equivalent, the resulting break in symmetry will depend on stochastic events in which the cell with the default state arises in a random fashion (Agrawal et al., 2009). But, if the initial cluster is nonhomogeneous (e.g., owing to prespecification by egg factors), such that the earliest cell to assume the default state is predetermined in its location, lateral inhibition will now yield a more predictable developmental outcome.

A last case in point, of many possible additional ones, involves the action of morphogens (the DPM termed "MOR"; Newman and Bhat, 2008, 2009). Here, a molecule secreted and released by one or more cells in a cluster diffuses or is otherwise transported throughout the aggregate, forming a gradient (Lander, 2007). Initially, equivalent cells can react differently to different concentrations of the morphogen, and thus assume different fates. Such a gradient will arise no matter what cell or cell group in the cluster serves as the morphogen's source—a robust outcome. It will take on a more reliable shape, however, if the source is prespecified (preset initial conditions), and even more so if one or more cells at other locations in the initial cluster have the capacity to absorb or break down the morphogen (preset boundary conditions).

Below, I describe how enlargement and reshaping of some ancient metazoan cells would have given rise to proto-eggs, in which preexisting intracellular processes generated spontaneous rearrangements of cytoplasmic molecules and materials. Once these large eggs cleaved into clusters, the resulting cells would have no longer been equivalent. The DPMs acting on these clusters would now have had their initial and boundary conditions preset, as described above. First, however, it is necessary to understand how primitive phylotypic body plans could have arisen in clusters of identical cells, in the absence of an egg stage.

THE "MORPHOGENETIC STAGE" AND THE ORIGINS OF ANIMAL EMBRYOGENESIS

The molecules involved in DPMs were already present before the appearance of multicellularity. As noted above, many of them are specified by genes present in the Choanozoa. The genetic similarity of these organisms to animals indicates that their common ancestor with metazoans, a hypothetical Precambrian unicellular form, already had the molecular capacity, when placed in the right context, to mobilize forces that could mediate the formation of multicellular clusters and then shape and pattern them.

The best characterized choanozoan, the choanoflagellate *Monosiga brevicollis*, contains several genes specifying cadherins, the family of proteins that mediate cell–cell adhesion in the embryonic tissues of all animal embryos, and which are thus the basis of metazoan multicellularity (Abedin and King, 2008). Choanozoan genomes also specify functional portions of the morphogen Hedgehog, as well as cell surface and intracellular components of the Notch pathway, which mediates lateral inhibition in metazoan embryos (King et al., 2008; Shalchian-Tabrizi et al., 2008). Although lateral inhibition is an inherently multicellular function, the Notch pathway may have evolved in single-celled organisms to perform the related role of influencing the choice between alternative cell states (Newman et al., 2009).

The Wnt pathway has two branches, one that mediates apicobasal cell polarity and the other planar cell polarity. Secretory Wnt proteins and their receptors have yet to be identified in a choanozoan, but they are present in the morphologically simplest metazoans, the sponges (Adell et al., 2007) and the only described placozoan, *Trichoplax adherens* (Srivastava et al., 2008). The fact that Wnt pathway components are also present in *Dictyostelium discoideum* (Dickinson et al., 2011), a protist more distantly related to the Metazoa than are the Choanozoa, suggests that the unicellular ancestors of the animals collectively had a fuller array of DPM-related toolkit genes than found so far in any one present-day choanozoan.

Some choanozoans have transiently colonial ecophenotypes. When considered along with their DPM-related toolkit genes, a plausible scenario emerges for the origin of developmental systems in the common ancestor of the choanozoans and metazoans. The first such systems would probably have been clusters of identical cells, possibly held together for prolonged times by preexisting cadherins taking on new cell–cell attachment functions owing to elevation of ambient Ca^{2+} (Kazmierczak and Kempe, 2004; Fernàndez-Busquets et al., 2009). These clusters would have been enabled for various modes of

morphogenesis by the DPMs that automatically came into existence once multicellular entities arose.

Not having the cell type heterogeneity and tissue polarity of the early stages of present-day animal embryos, these ancient clusters would nonetheless have been capable of undergoing processes resembling gastrulation, elongation, segmentation, and so forth, only not as reliably as modern metazoans (Newman and Bhat, 2008, 2009). Despite the variability of their expression, the morphological motifs that arose from the action of DPMs in these ancient cell clusters remained constant themes in the generation of body plans and organ forms over the last half billion years.

The hypothesized premetazoan cell colonies could have propagated their morphotypes in a similar fashion to the social amoeba *D. discoideum*, described above. Because the genome(s) of the constituent cells would have contained a collection of DPM-associated toolkit genes, any one of them, if detached from an existing cluster, would have been capable of founding a new cluster with the same self-organizing capabilities. By simply dividing and aggregating (or remaining attached to one another), the progeny of the founder cell would reconstitute the same set of DPMs (i.e., the genes plus the physical context and associated processes) present in the earlier cluster. In this sense, the founder cell would serve as the prototype of a zygote.

It should be emphasized that the described period of metazoan evolution is a primitive one, before the existence of developmental programs, organismal individuality, or populations in the sense usually considered in evolutionary models (Newman, 2005; Newman et al., 2006). Although DPMs would have been capable or organizing clusters of cells into entities with animal-like bodies, these were far from being like present-day animals. What was needed for the transition from ancient to modern metazoans was a set of mechanisms for increased reliability in the generation of form.

As noted, DPMs are formed by the harnessing of specific physical processes by certain products of the ancient developmental-genetic toolkit, on the scale and context of the multicellular state. The detailed outcomes mediated by these physical processes are highly dependent on the system's boundary conditions (including shape) and the initial conditions (spatially dependent concentrations, binding strengths) of the key system variables (e.g., morphogens, adhesivity). Appropriate sets of DPMs can organize clusters of cells into forms containing the morphological motifs of all the extant metazoan body plans. But, in the absence of prespecified boundary and initial conditions, the polarity, symmetry or asymmetry, and morphological details of otherwise equivalent modules (e.g., segments, appendages) generated by these patterning mechanisms would not be well regulated (Newman and Bhat, 2008, 2009).

Were these early forms animal body plans or at least prototypes of them? The term body plan has several common, but sometimes conflicting, meanings. Wray and Strathmann list four of these: (i) distinguishing morphological characteristics of a phylum or class; (ii) architectural features of animals; (iii) the traits of a phylotypic stage; and (iv) patterns of gene expression in embryos (Wray and Strathmann, 2002). From the discussion above, the DPM-based conception of body plans integrates at least the first three of these, and to a certain extent the fourth. DPMs incorporate members of the conserved developmental-genetic toolkit, not all of which are present in each animal phylum. Because particular toolkit components can mobilize distinctive physical effects, a phylum's or class's complement of DPMs has a major influence over the morphological characteristics and the architectural features of its members. These features have a genealogical basis owing to the "phylogenetic stratification" (Domazet-Lŏso and Tautz, 2010), i.e., order of evolutionary appearance, of the genes the DPMs incorporate.

In contrast to notions of a phylotypic stage characterized by anatomical descriptors (e.g., germ-band, pharyngula) or patterns of gene (e.g., Hox) expression, the "morphogenetic stage" at which DPMs become operative once cleavage is completed (depending on the species, the morula, blastula, blastoderm, or inner cell mass; see also Seidel, '60) is defined by a set of pattern-forming capabilities arising from the particular DPMs available to the cell aggregate (Tables 1 and 2). Although the morphologically characterized phylotypic stage will typically be consequential to, and thus somewhat later in development than the morphogenetic stage, it is expected that the embryonic period that includes both of these will manifest expression of the most ancient and conserved developmental genes (Domazet-Lŏso and Tautz, 2010; Kalinka et al., 2010). There is no need, in this view, to attribute this constrained pattern to the stabilizing effects of natural selection as proposed in the cited studies.

EGGS AS EVOLUTIONARY INNOVATIONS AND MORPHOLOGICAL NOVELTIES

Once primitive animal body plans had emerged, there would have been a selective premium on starting the developmental sequence not with a cluster of developmentally equivalent cells, but with a cell aggregate that was "prepared" so that development would proceed in a more reliable fashion. Generally accepted scenarios predict that changes made at the earliest stages of development should have dramatic consequences at later stages. The fact that such varied changes can occur even in related lineages during the period leading up to gastrulation, with little effect on the body plan, is the crux of the (preconstriction) hourglass puzzle. The DPM perspective accounts for this phenomenon in a natural fashion without recourse to ad hoc selectionist arguments. Specifically, the intrinsic variability in developmental outcome (within the limits of the associated physical processes), when a set of DPMs acts on a cluster of equivalent cells, would actually be suppressed or reduced when the same DPMs organize a cluster of cells that is rendered axially polarized or otherwise asymmetric by earlier acting processes. Furthermore, in contrast to cell clusters generated by aggregation of choanozoan-like cells, cell clusters produced by subdivision of proto-eggs would have been genetically uniform,

contributing to reliable intergenerational propagation of type (cf. Grosberg and Strathmann, '98). Distinctive egg stages thus represented evolutionary innovations that enabled the formation of genetically stable and developmentally stereotypical variants within previously roughhewn phylotypes.

Eggs differ most obviously from blastomeres in size and shape. The best-characterized molecular regulator of animal cell volume is the Na^+,K^+ ATPase (Stein, 2002). Although the α-subunit of this enzyme is partially specified in the *M. brevicollis* genome, the corresponding proteins lack certain key properties of the metazoan version, and there are no genes for the β-subunits of the protein complex important for the volume regulatory function (Sáez et al., 2009). Cell shape in eukaryotes is largely under the control of a highly structured cortical actin cytoskeleton (Morris, 2002; Lecuit and Lenne, 2007). Although 1.5 billion year old protistan microfossils show evidence of a cytoskeleton-based architecture, they are up to 160 μm in diameter (Javaux et al., 2001) as compared with the 3–10 μm diameter of choanoflagellates. The surface appearance of the microfossils suggests that their regulation of cell morphology "lacked the finesse of modern cells" (Morris, 2002).

Therefore, even if the choanozoan–metazoan ancestors, which first aggregated and became organized by DPMs, had already evolved size and shape regulatory mechanisms similar to those of modern animal cells, they were probably only a few mutations away from cellular expansion and reshaping. Moreover, cells shed from the premetazoan clusters and capable of multiplying and regenerating the latter would also, in isolation, have been relatively more susceptible to microenvironmental modulation of their size and shape. Adaptive selection favoring the accumulation of stored energy-rich molecules (i.e., yolk) would have contributed to the further enlargement and reshaping of these proto-eggs.

It is important to emphasize that, in the scenario described here, egg forms or types could have a variety of causes and represent distinct kinds of adaptation. Proto-eggs for the different phyla may have arisen entirely independently of each another, whereas at the subphylum level different types of egg may represent transformations of one another. In any case, a combination of adaptive and nonadaptive processes would have contributed to the egg's form. The consequences of these morphological variations for the organization of the egg's interior are a separate question, discussed in the following section.

EGG-PATTERNING PROCESSES

Oocytes and pre- and postfertilized eggs are rendered internally nonuniform by two kinds of processes. The first, common to all animal taxa except for a few (e.g., eutherian mammals) are cytoplasmic determinants ("ooplasms") incorporated into distinct regions of the egg during oogenesis. Because this article is concerned with the origin of the egg stage of development in premetazoan cell aggregates, before the existence of animal bodies containing gonads and gametogenesis, this mode of internal egg patterning will be put aside for the moment.

The second mode is mediated by a set of physical and physicochemical effects (e.g., diffusion and sedimentation gradients, calcium ion transients, phase separation of cytoplasmic factors), induced by sperm entry or parthenogenetic activation. The cytoplasmic heterogeneities generated by either or both modes, though often associated with recognizable polarities and landmarks of the adult stage, in most cases do not correspond to maps or blueprints of the developed organism, or even of intermediate embryonic stages (see, for example, Freeman, '99).

As described in the examples below, instances of the second mode, EPPs, are built upon single-cell cytoplasmic functionalities that evolved before the existence of eggs, and indeed of multicellularity. In most cases, the relevant intracellular processes have some "generic" physical aspects, in the sense that they are based on material properties and capabilities, such as diffusion, viscoelasticity, sedimentation, and convection, which are common to living and nonliving systems (Newman and Comper, '90). Some molecular commonalities in EPPs are also undoubtedly owing to deep homologies in intracellular polarization mechanisms that even preceded the opisthokont (e.g., choanozoa) amoebazoan (e.g., social amoeba) split (Dickinson et al., 2011). In almost every instance, however, these EPPs have evolved so that their outcomes are supported or reinforced by additional molecular machinery.

Under the hypothesis presented here, EPP-induced nonuniformities, though developmentally consequential, are not primarily adaptations, in the sense of having been gradually arrived at through multiple cycles of selection, in response to external "pressures." (This is in contrast to the egg size and shape modifications that set the stage for them, which were very plausibly adaptive.) The likely nonadaptive origin of many ooplasmic nonuniformities is owing to the fact that the generic physical components of the EPPs have inherent propensities to organize matter in preferred directions, and to sometimes do so in abrupt nonlinear fashion in response to changes in system parameters. This limits the degree to which the outcomes can be incrementally molded by selection.

Some of the variant organisms that result from DPMs operating with initial and boundary conditions established by the action of EPPs, however, are likely to have been more ecologically successful than others, so that (as with the body plan-determining DPMs) evolutionary change would have been partly driven by saltational processes. Several EPPs operative in different phyla, exhibiting a variety of interactions among molecular and generic physical components, are described in detail in the following sections.

Long Germ-Band Insects: Intracellular Diffusion Gradients and Self-Organization

As seen above, morphogen gradients can be established across multicellular aggregates by the process of molecular diffusion in

the extracellular space. Within single cells, however, the crowded cytoplasm curtails the effectiveness of free diffusion as a transport mechanism and as a generator of informational gradients (Agutter and Wheatley, 2000). It has been proposed, however, that rapid transport through the cytoplasm can occur by a kind of "reduced-dimension" diffusion of signaling molecules along interconnected networks of cytoskeletal filaments (Forgacs, '95; Shafrir et al., 2000).

Even if diffusion is thus facilitated, the linear dimension of an individual cell is so small (~10 μm) that diffusion gradients would tend to flatten over relatively short times. This is not necessarily the case for eggs, however, which can be much larger than somatic cells. Most *Drosophila* species have eggs on the order of 500 μm in length (Markow and O'Grady, 2005) and eggs of other long germ-band insects range up to 1.4 mm (Gregor et al., 2005). *Drosophila* eggs have maternal mRNA specifying the transcription factor Bicoid stored in their anterior ends during oogenesis. Bicoid mRNA associates with the protein Staufen and is transported by the reduced-dimension diffusion mechanism described above, along a microtubule network that pervades the egg cortex, forming a concentration gradient (Spirov et al., 2009) (Fig. 2A).

Because its mRNA diffuses to form an anteroposterior (A-P) gradient, the Bicoid protein distributes in a corresponding fashion, with correspondingly different amounts freely entering the approximately 5,000 egg nuclei which (having been generated by endomitosis) are not yet enclosed by membranes (Gregor et al., 2007). Bicoid and other maternal factors induce the expression of "gap" transcription factors and these in turn induce

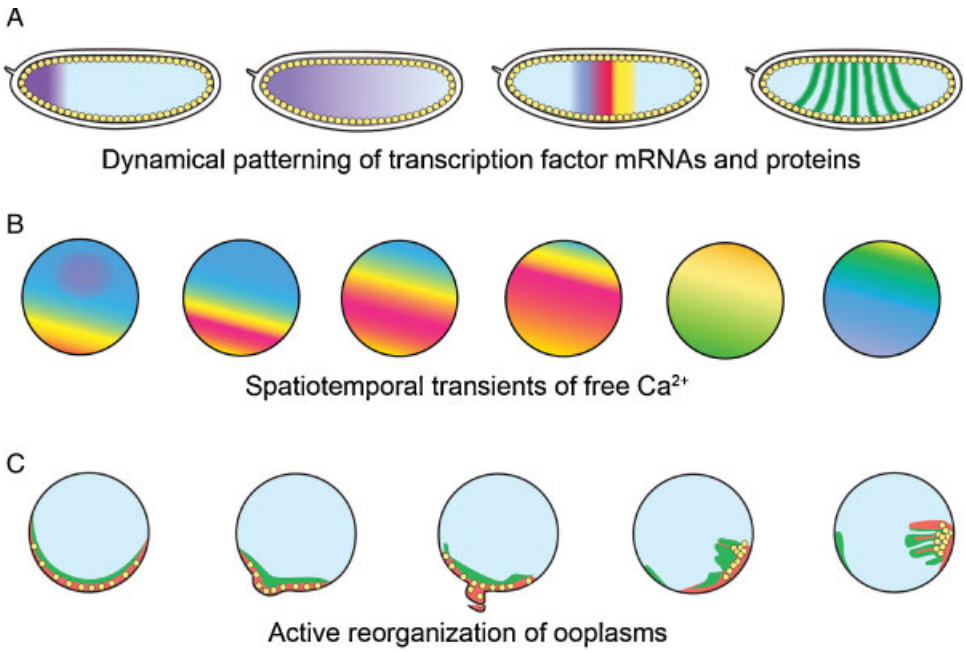

Figure 2. Schematic representation of different types of EPP. (**A**) Generation of molecular patterns in the *Drosophila* egg cytoplasm before cellularization of the blastoderm. The first panel shows the distribution of Bicoid mRNA (purple) in the newly laid egg. This is a maternally deposited pattern not owing to an EPP, but is transformed by an EPP, cytoskeleton-facilitated diffusion, into the concentration gradient seen in the second panel. The resulting gradient of Bicoid protein initiates a series of local autoactivation and lateral inhibition (LALI) involving the regulation of gap and pair-rule genes and their products (third and fourth panels) in the excitable medium constituted by the layer of unsequestered nuclei (small circles). See text for references. (**B**) Patterns of spatiotemporal Ca^{2+} waves in a fertilized ascidian egg, visualized under confocal microscopy with a Ca^{2+}-sensitive dye. Release of Ca^{2+} from intracellular stores is imaged, with warmer colors corresponding to higher concentrations of free Ca^{2+}. Fertilization has occurred at the lower right of the first image, and the whole wave propagation sequence occurs for more than 72 min. The calcium wave EPP is associated with cytoplasmic reorganization and other molecular changes in several phyla (see text and Table 3). Drawing based on photographs in Dumollard et al. (2002). (**C**) Ooplasmic reorganization in an ascidian egg between fertilization and the start of cleavage. The site of fertilization in this case is the upper right quadrant of the left-most panel, and the cytoplasmic flows and contractions leading to the changes are associated with Ca^{2+} waves like those shown in (B). Yellow granules represent "postplasmic" stored maternal messenger RNAs (PEM); material shown as red is cortical endoplasmic reticulum; green represents "myoplasm," containing mitochondria and vesicular inclusions. Further characterization of the reorganizing components can be found in Sardet et al. (2007), from which this series was redrawn.

the production of "pair-rule" transcription factors (Fig. 2A; see St Johnston and Nusslein-Volhard, '92; Lawrence, '92; for reviews).

By the time the nuclei become enclosed in membranes (forming the cellular blastoderm), the distribution of the various pair-rule transcription factors exhibits a strict spatial periodicity that provides the basis for the eventual morphological segmentation of the insect body. The nonuniform distributions of the pair-rule gene products (even-skipped, fushi tarazu) emerge, in part because of the presence of dedicated promoters with positive and negative responses to different concentrations and combinations of Bicoid or gap proteins, leading to stripes of the corresponding mRNAs at specific locations along the egg's long axis (St Johnston and Nusslein-Volhard, '92).

Local auto activating and lateral inhibitory (LALI; Meinhardt and Gierer, 2000) interactions among pair-rule and gap factors are also involved in establishing pair-rule stripes (Harding et al., '89; Schier and Gehring, '93; Yu and Pick, '95; Clyde et al., 2003). Here, the layer of syncytial nuclei, being directly responsive to the diffusing transcription factors that they collectively specify, constitute what is known as a (chemically) "excitable medium" (Mikhailov, '90) (Fig. 2A). Such self-organizational effects were plausibly in the main modes of egg patterning early in long germ-band insect evolution, before gene duplication led to position-dedicated promoters (Salazar-Ciudad et al., 2001).

It should be noted in relation to the present hypothesis that the cellular blastoderm, which is the DPM-capable morphogenetic stage of long-germ band insects, contains a repetitively patterned, rather than uniform, distribution of cells at the time the multicellular morphogenesis gets underway. As seen earlier, this initialization of the segmentation process arises by the spontaneous action of cytoskeleton-mediated diffusion and transcriptional activator–inhibitor interactions in the responsive medium represented by the layer of nonsequestered nuclei on the expanded (relative to a typical cell) scale of the egg.

Finally, it is significant that the long germ-band mode of development is most likely to have been derived from the short germ-band mode. In the cellular embryos of the ancestral forms, DPMs generate a virtually identical segmental pattern, but by successive addition of segments from a growth zone rather than by utilizing a cytoplasmic prepattern (reviewed in Salazar-Ciudad et al., 2001). This thus seems to be a case in which a generation of successful morphological motif by DPMs (in short germ-band forms) has been displaced to the egg stage of development (in long germ-band forms) by employing EPPs.

Such a heterochronic shift would not have been trivial. Although intermediate germ band insects utilize the "sequential" mode in one portion of the embryo and the "simultaneous" mode in another (Rohr et al., '99), this does not represent the kind of incremental intermediate between forms envisioned by adaptationist scenarios. The transition was probably facilitated by the appearance of Bicoid, a novel gene (Stauber et al., '99), whose mRNA could be redistributed by existing physical means (cytoskeleton-dependent diffusion) and the effect (i.e., an A-P gradient) of this redistribution in the novel physical and geometric context of the egg. But, the key enabling determinant of the embryo-to-egg shift in the initiation of segmentation was probably the self-organizational dynamics of previously existing ingredients (e.g., pair-rule genes and their products) that emerges when the proteins operate in a context (i.e., a syncytium) in which they can freely diffuse (Salazar-Ciudad et al., 2001).

Ascidians: Calcium Waves and Cytosolic Reorganization

In many animal taxa, including echinoderms, amphibians, and mammals, as soon as fertilization takes place the egg's concentration of intracellular calcium ion released from internal stores increases approximately ten-fold. This occurs (depending on the species) in the form of one or more traveling waves of elevated Ca^{2+}, which start at the point of sperm entry and propagate across the egg. Although the initial effect of the elevated calcium ion concentration is the triggering of the cortical granule reaction, which establishes the slow block to polyspermy (Jaffe et al., 2001), the subsequent waves cause additional major intracellular restructuring that establishes positional determinants for later development (Dumollard et al., 2002).

The ascidian egg (e.g., *Ciona intestinalis*, but similarly *Phallusia mammillata* and *Halocynthia roretzi*) contains three distinct Ca^{2+} wave pacemakers (Dumollard et al., 2004), one of which is pictured in Figure 2B. The generation and propagation of these waves occurs when the cytosolic concentration of calcium ions reaches a threshold value, inducing the release of sequestered calcium ions, a phenomenon known as Ca^{2+}-induced Ca^{2+} release (Jaffe, 2008). At the threshold, according to a widely accepted model, inositol trisphosphate (IP_3)-responsive receptor channels in the intracellular compartments are opened. These channels are inactivated as the local Ca^{2+} concentration further rises, remaining closed during a refractory period. Finally, cytosolic Ca^{2+} is resequestered into the endoplasmic reticulum via specialized pumps (Kline and Kline, '92).

All these Ca^{2+} regulatory components, including plasma membrane- and intracellular store-operated Ca^{2+} channels and IP_3 receptors, are present in somatic cells. Significantly, they are also found in the choanoflagellate *M. brevicollis* (Cai, 2008), and were therefore a likely part of the signaling repertoire of the unicellular ancestor of the metazoans. In neurons and smooth muscle cells of advanced metazoans, this set of functionalities can mediate temporal oscillations in Ca^{2+} concentration (Mikoshiba, 2007; Berridge, 2008), and this capacity was plausibly present in single-celled ancestors. But, as is well-known from the analysis of physical systems, an increase in domain size (as in the evolutionary cell-to-egg transition) can transform the behavior of a dynamical system from spatially uniform temporal oscillations to spatially nonuniform traveling waves, such as those seen in ascidian eggs (Aihara and Yoshikawa, 2001).

The described Ca^{2+} pacemakers in ascidian eggs and the cortical mechanical waves that accompany them are associated with, and may be the drivers of, a series of dramatic reorganization of cytoplasmic components, including maternal RNAs, mitochondria, and endoplasmic reticulum, which follow (Sardet et al., 2007) (Fig. 2C). During the 1 hr between fertilization and the first cleavage, a single calcium wave, followed by a microfilament-dependent cortical contraction and a series of repetitive calcium waves, is associated with the relocation of several cytoplasmic determinants of body axis organization, gastrulation, and organogenesis produced before fertilization. The future dorsoventral axis is determined during this period. Some of the cytoplasmic determinants, which contain mRNAs and mitochondria in addition to proteins, translocate along the cortex during a subsequent microtubule-dependent phase in which the cortex and egg surface vibrates in a rapid fashion. Then, during a new microfilament-dependent phase, the cortex relaxes and the determinants of the future A-P axis assume their definitive positions (Sardet et al., 2007). One of these determinants, an RNA molecule known as PEM, also regulates the embryonic expression of fibroblast growth factor and suppresses the competence of certain blastomeres to respond to this morphogen, thus restricting notochord and brain induction to appropriate regions (Kumano and Nishida, 2009).

As with the long germ-band insect case described above, the eggs of ascidians sustain a number of dynamical processes which, by reorganizing molecular species and cytoplasmic complexes, set up the initial and boundary conditions in the form of prespecified cell states for the operation of DPMs, once cleavage generates the morphogenetic stage. Whether the basis of these rearrangements is (as suggested by the hypothesis described here) spontaneous manifestations of single cell activities that take novel forms in the expanded space of the egg or (as the default account in the standard evolutionary framework would suggest) incrementally selected adaptations is an open question. A third example of the action of EPPs in still another phylum provides additional insight on this issue.

Nematodes: Polar and Nonpolar Routes to a Conserved Morphological Endpoint

The egg of the nematode *C. elegans* (unlike those of the insects and ascidians described above) is unpolarized before it is fertilized. Sperm entry triggers the reorganization of the egg's cortical cytoplasm before the first cleavage, leading to an asymmetric distribution of various factors, which is required for the establishment of the A-P axis during embryogenesis. Cortical reorganization begins with transient focal actomyosin-driven contractions throughout the cortex. The sperm microtubule organizing center (MTOC) causes an asymmetrical contraction of the remaining actomyosin network, leading to a flow of cortical actomyosin toward the future anterior pole. These flows cause the enrichment in an anterior cap of the initially uniformly distributed complex of the enzyme Pkc-3 with the two scaffold proteins Par-3 and Par-6 and their depletion from the posterior cortex. This, in turn, releases the serine–threonine kinase Par-1 to accumulate in a complementary posterior domain of the cortex. In this way, although the sperm does not attach at a preferred site on the egg, its entry point defines the future posterior pole (Munro et al., 2004; Munro, 2007; Tsai and Ahringer, 2007). The described mechanism suggests the action of an EPP analogous to that seen in ascidians (Fig. 2C); that is, ancient cell dynamics (e.g., cortical flows; Paluch et al., 2006) mobilized in new ways in the context of the enlarged egg cell.

It is possible that A-P polarity in *C. elegans* evolved by a selection regime that refined the organization of molecules in the egg cytoplasm, so that the mature nematode body could attain a functionally well-adapted form. Alternatively, if the nematode body plan is (as hypothesized here) a necessary outcome of the action of the characteristic DPMs that operate at the morphogenetic stage of these organisms, the means by which A-P symmetry is broken and even the pregastrulation stage at which it occurs, would have relatively little impact on the final form.

The second of these possibilities gains plausibility from the fact that the nematode *Bursaphelenchus xylophilus*, which is anatomically indistinguishable from *C. elegans*, generates its A-P polarity in an entirely different fashion from the latter. In *B. xylophilus*, the sperm entry point becomes the future *anterior* pole of the embryo, and the pattern of cortical flow and its relation to the MTOCs are entirely different from that in *C. elegans* (Hasegawa et al., 2004). In another nematode, *Romanomermis culicivorax*, the first cleavage is symmetric rather than asymmetric, and the pattern of asymmetric cleavages and alternative assignment of cell fates suggests that A-P polarity is determined in still a different fashion from the other two nematode species (Schulze and Schierenberg, 2008). In the freshwater nematode, *Tobrilus diversipapillatus*, no asymmetric cleavages and no distinct cell lineages are generated until the morphogenetic stage, which resembles that of all nematodes previously studied, but in contrast to them begins with a hollow blastula typical of other metazoans instead of a solid ball of cells (Schierenberg, 2005). Finally (although many more instances could be listed), in three different parthenogenetic species of nematodes, with no opportunity for sperm entry to influence the assignment of A-P polarity, this essential developmental feature is acquired in ways that not only differ from *C. elegans*, but also from one another (Lahl et al., 2006).

To summarize, although acquisition of A-P polarity is clearly an essential feature of nematode anatomy, the way that it is acquired during development seems to have little impact on the final morphological outcome, which is always, apart from size, remarkably similar. The variability of the symmetry-breaking processes, moreover, raises the possibility that mechanisms other than adaptive evolution are responsible for its many manifestations. If the common morphological outcome resides in the

Table 3. Major egg-patterning processes (EPPs) and their presence in various genera.

EPP	Genus
Cytoskeleton-facilitated diffusion	*Drosophila*
Chemically excitable medium composed of syncytial nuclei	*Drosophila*
Ca^{2+} spatiotemporal transients	*Lytechinus* (sea urchin); *Ciona*, *Xenopus*; *Mus*
Viscoelastic cortical and deep ooplasmic flows	*Caenorhabditis*; *Ciona*
Gravitational sedimentation+ cytoskeleton-mediated ooplasmic flows	*Xenopus*

nematode-characteristic DPMs that operate at the morphogenetic stage, the EPPs and the premorphogenetic stage cell–cell interactions they facilitate will play subsidiary, boundary value- and initial condition-setting roles. Some major EPPs and organisms in which they appear are listed in Table 3 and depicted schematically in Figure 2.

EPPS AND THE VERTEBRATE HOURGLASS

No animal taxon has a wider variety of egg morphologies and early developmental routes than the vertebrates, making this subphylum the paradigmatic case of the embryonic hourglass (Raff, '96). Members of all the vertebrate classes, including (nonplacental) mammals, have unusually yolky eggs, a property that clearly coevolved with the capacity to sustain development outside the maternal body. Yolk, being denser than cytosol, tends to sediment under the force of gravity, which provides the physical aspect of some EPPs (see below). Once internal gestation evolved, the vertebrate egg was free to revert to a small, nonyolky condition, which is seen in all placental mammals.

The eutherian mammalian (e.g., mouse) egg becomes internally organized postfertilization by an EPP, in this case a set of Ca^{2+} transients similar to but even more complex than that described above for the ascidian egg (Fig. 2B). Several important postfertilization events in the mouse (cortical granule exocytosis, cell cycle resumption, and recruitment of maternal mRNAs) are initiated by different numbers of Ca^{2+} waves. The completion of each such event requires a greater number of Ca^{2+} waves than its initiation (Ducibella et al., 2002). In both fertilized and parthenogenetically activated eggs, the presence or absence of specific patterns of Ca^{2+} oscillation influence later patterns of zygotic gene expression (Ozil et al., 2006; Rogers et al., 2006).

The zygote must have a mechanism to "interpret" patterns of Ca^{2+} oscillations so as to affect later embryonic events. In mammals, this seems to partly involve the ancient (Ohya et al., '91) enzyme Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), which oscillates in concert with postfertilization Ca^{2+} waves (Markoulaki et al., 2003, 2004) and plays a central role in regulating precleavage events, such as cortical granule exocytosis, and exit from meiotic metaphase II/resumption of the cell cycle (Abbott and Ducibella, 2001; Tatone et al., 2002; Malcuit et al., 2006). This same enzyme responds to very high frequency Ca^{2+} pulses in neurons (De Koninck and Schulman, '98), though in the egg it tracks the calcium signal more closely. This is another example of an EPP, where a single-cell dynamical process seems to have taken on new properties in the altered geometric context of the egg, although incremental adaptive effects may have also played a role in refining these responses.

As with the representatives from other phyla (e.g., arthropods, nematodes, and ascidians) discussed in previous sections, experiments show the importance of mammalian EPPs in the fidelity of developmental outcomes, though not necessarily in the generation of the major features of body plan. When normal patterns of Ca^{2+} oscillations were perturbed, interrupted, or circumvented in fertilized mouse eggs, implantation rate and pre- and postimplantation embryo growth rates were all impaired (Kurokawa and Fissore, 2003; Ozil et al., 2006; Rogers et al., 2006).

As noted, gravity acting on ooplasmic components also mediates an EPP in the precleavage frog egg. Brief reorientation of the egg to the gravitational field after fertilization causes abnormal body patterns in the resulting larvae, indicating that normal orientation is used as positional cue (Cooke, '85; Vincent and Gerhart, '87). Although the dorsoventral orientation of the *Xenopus* embryo is determined by such gravity-driven rearrangements, in keeping with some other EPPs described above they are not essential for a normal developmental outcome (Neff et al., '83, '84). Their roles in fine-tuning or fidelity of development have nonetheless apparently been important enough for them to have come under the control of more active and molecularly complex processes than simple gravity, such as polarized transport (Danilchik and Gerhart, '87) and surface contraction waves (Denegre and Danilchik, '93).

An additional postfertilization rearrangement of ooplasmic components, known as cortical rotation, is also normally required for specification of the dorsoventral axis in anurans. This rearrangement depends on microtubule-based motors (Houliston and Elinson, '91). A ubiquitin ligase, trim36, which mediates this effect, is disrupted (along with the microtubule polymerization that drives it) in trim36-depleted eggs. Nonetheless, tipping the egg 90° relative to the animal–vegetal axis rescues the embryos and restores normal development (Cuykendall and Houston, 2009). Thus, although cortical rotation itself is dispensable for body axis formation, it sets up intracellular conditions that help it to occur reliably (Vincent and Gerhart, '87; Weaver and Kimelman, 2004).

In view of the variability, plasticity, and redundancy of mechanisms of precleavage egg patterning in vertebrates, I suggest that the role of EPPs in body plan development of these organisms is modulatory rather than essential. This secondary role of EPPs is owing to the vertebrate body plan being shaped by

subphylum-associated processes acting at the multicellular scale, i.e., DPMs. Little that may occur during the egg stage to cause the morphogenetic stage cells to assume specialized identities will change which DPMs become activated at that later stage. This is why egg size and shape can vary over wide ranges, and the fertilized egg cytoplasm can become extensively reorganized by nonlinear, conditional, dynamical processes (which are unlikely to have been subject to incremental adaptive selection) without marked consequences to the vertebrate body plan.

CONCLUSIONS

The foregoing discussion can be summarized in the following five points:

(i) The phylotypic body plan of an organism is defined by the DPMs (consisting of molecules *and* physical effects) inherited by its cells rather than by specific spatial patterns of gene expression.
(ii) Phylum-associated morphological motifs are generated beginning at the *morphogenetic stage*, when an organism's embryo consists of a cluster of relatively equal-sized cells.
(iii) The origination of each of the animal phyla occurred via the aggregation of choanozoan-like cells containing overlapping but distinct sets of DPMs. The simple reproductive cycles of these proto–phyla would have been achieved by the shedding of cells and their reaggregation into morphogenetic stage clusters.
(iv) The first eggs arose when shed cells from ancient phylotypic clusters became enlarged and reshaped, so that spontaneous internal self-organizational processes (EPPs) were unleashed. Cleavage type subdivision now regenerated morphogenetic stage clusters, in which cells differed in their states according to various stereotypical patterns rather than being all the same.
(v) Because the DPMs were identical for organisms in a given evolutionary lineage before and after the acquisition of an egg stage, phylotypes were unchanged by the emergence of eggs. Organisms that develop from egg-derived morphogenetic stages, however, exhibit reliable variations on phylotypic anatomies, because the characteristic DPMs now operate with consistent boundary and initial conditions rather than having to depend on stochastic symmetry-breaking effects.

By applying different criteria and methods, a number of other investigators have argued for (Slack et al., '93; Duboule, '94; Raff, '96) and against (Wray and Strathmann, 2002; Bininda-Emonds et al., 2003) the existence of a phylotypic stage and the objectivity of the embryonic hourglass heuristic. The proposal described here reconciles these views by shifting attention to the DPMs and the morphogenetic stage. Given what the DPMs do—generate forms that are variously hollow, multilayered, folded, elongated, segmented, etc—it makes sense that the presence of a phylum-specific set of them will produce organisms having a "family resemblance." Because the DPMs generally begin to operate when a critical mass of cells is present (i.e., the morphogenetic stage), members of the same phylum will typically continue to resemble one another in the period following that stage, although the embryos of certain phyla (e.g., molluscs) can exhibit much greater diversity in morphological outcome than those of others (e.g., nematodes) (Salazar-Ciudad, 2010). As the embryos of a given phylum develop beyond the morphogenetic stage, they will pass through some stages at which the phylum-typical morphological motifs begin to manifest themselves (e.g., the insect germ-band stage, the vertebrate pharyngula). Thus, although the phylotypic stage (defined morphologically) and the hourglass format will often be only approximate (Fig. 1B), they are not thereby "subjective" (cf. Bininda-Emonds et al., 2003).

The lower portion of the hourglass is constituted by the requirement to reconstitute the morphogenetic stage from the fertilized egg so that the DPMs can be set in motion. The EPPs, which came into existence with the evolutionary appearance of enlarged or elongated premetazoan founder cells, generate cytoplasmic heterogeneities that are conveyed to the cell clusters derived from them in the form of patterned arrangements of blastomeres. The "autonomous" (i.e., single-cell; Salazar-Ciudad et al., 2003) patterning mechanisms that produce asymmetric morphogenetic stage clusters, whether following from the self-organizational properties of EPPs or, later in evolution, from the maternal deposition of ooplasms, do not change the array of DPMs available to the taxonomic lineage. Thus, the insertion of highly variant egg stages into the developmental trajectory of phylum or subphylum would not have altered its phyletic nature.

This interpretation of the hourglass phenomenon implies that temporal gene expression patterns for different species within the same broader taxonomic group should be maximally conserved mid-way through embryogenesis, when the DPMs are operative rather than at earlier or later stages. An even more counterintuitive implication of this model is that when compared across phyla, the evolutionarily oldest genes should be expressed not at the egg stage but later during development at or around the "phylotypic" stage. Both these predictions have been confirmed in recent studies (Domazet-Lõso and Tautz, 2010; Kalinka et al., 2010).

The step in the evolution of eggs I have focused on here is an early one, relating to the capacity of enlarged nutrient-rich cells to undergo cleavage and regenerate the body plans of the haploid metazoan ancestors from which they arose. Their functionality as gametes presumably came later, with the recruitment of syngamy and meiosis (the ingredients for which are present in choanozoans; Carr et al., 2010). Perhaps the most striking implication of the analysis presented here is the suggestion that eggs were novelties that appeared after the rise of metazoan phyla from ancestral choanozoan clusters. Chickens and their eggs, being rather late arrivals in taxonomic terms, must have coevolved in their specific properties. But, the perennial question of "which

came first," applied to a primordial chordate and its egg, must by the proposed hypothesis be decided in favor of the chordate.

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